Manuscript Title

Prominent oncogenic roles of EVI1 in breast carcinoma

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Running Title: EVI1 in breast carcinoma

Keywords: EVI1, breast cancer, MAPK, KISS1

Financial support: This study was performed as contracted research supported by the Baden-Württemberg Stiftung (Adult Stem Cells Program II) and by grants from the Deutsche Forschungsgemeinschaft to S.P. (PE 1179/4-1) and the Schweizer Nationalfonds to C.L. (310030_149735).

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Disclosure of any potential Conflicts of Interest

The authors declare no conflicts of interest regarding this publication.

Abstract

Overexpression of the EVI1 oncogene is associated typically with aggressive myeloid leukemia, but is also detectable in breast carcinoma (BC) where its contributions are unexplored. Analyzing a tissue microarray of 608 BC patient specimens, we documented EVI1 overexpression in both estrogen receptor-positive (ER+) and estrogen receptor-negative (ER-) BC. Here we report prognostic relevance of EVI1 overexpression in triple-negative BC (TNBC) but not in the HER2-positive BC subset. In human breast cancer cells, EVI1 silencing reduced proliferation, apoptosis resistance and tumorigenicity, effects rescued by estrogen supplementation in ER+ BC cells. Estrogen addition restored ERK phosphorylation in EVI1-silenced cells, suggesting that EVI1 and estradiol signaling merge in MAPK activation. Conversely, EVI1 silencing had no effect on consitutive ERK activity in HER2+ BC cells. Microarray analyses revealed G-protein coupled receptor (GPR) signaling as a prominent EVI1 effector mechanism in BC. Among others, the GPR54-ligand KISS1 was identified as a direct transcriptional target of EVI1, which together with other EVI1-dependent cell motility factors such as RHOJ regulated BC cell migration. Overall, our results establish the oncogenic contributions of EVI1 in ER- and HER2-negative subsets of breast cancer.

Introduction

Breast carcinoma (BC) is the most common malignant tumor and predominant cause of cancer-related death in women worldwide. During the last years, increasing BC heterogeneity has been documented concerning mutational background, histopathology, dissemination patterns and efficacy of surgical, antihormonal, chemo- or radiation therapies. Despite high initial remission rates especially in early stage disease, BC patients carry a significant life-long risk for disease relapse (1). Recent research has focused on so-called breast carcinoma stem cells (CSCs) as mediators of tumor relapse after long latency (2) as well as on stemness proteins as CSC biomarkers and potential drug targets (3,4).

The *EVI1* gene is part of the complex MECOM locus on human chromosome 3q26 and encodes a zinc finger transcription factor that is expressed in long-term repopulating hematopoietic stem cells (HSCs) (5,6). In acute myeloid leukemia (AML), *EVI1* overexpression can occur due to chromosomal rearrangements or as a reflection of the stem cell origin of the disease, but in either case predicts very adverse prognosis (7). *EVI1* expression has been also reported in solid tumors including breast carcinoma (8,9), where it is still largely understudied with respect to relevance, functional roles and molecular regulation.

Here we performed a comprehensive expression and functional analysis of *EVI1* in human BC. By analyzing a tissue microarray of 608 patient samples, we found high EVI1 protein expression in BC regardless of the ER status. A detailed clini-

co-pathological investigation uncovered a prognostic significance of EVI1 expression in ER- and especially triple-negative BC, which was however not observed in HER2+ tumor subsets. While EVI1 depletion impaired cell cycle progression, apoptosis resistance and MAPK signaling in both ER- and ER+ BC cells, addition of estrogen could rescue these effects only in ER+ cells. Moreover, similar as in patients, HER2 overexpression appeared to overrule EVI1 effects on MAPK signaling, explaining why EVI1 expression is of particular clinical relevance in the ER- HER2- tumor subset. Finally, we identified the GPR54-ligand KISS1 as a novel transcriptional target of EVI1, which promotes BC cell migration. In sum, our report identifies *EVI1* as an oncogene that profoundly regulates BC biology and that is of particular importance for estrogen-independent HER2-negative tumors.

Materials and Methods

Human tumor samples and tissue microarray (TMA) analysis

Handling of patient samples and data analyses were performed in accordance with federal and state laws and approved by the local ethics committee. The TMA included samples from 608 human primary BC (primary or recurrent) histologically processed and diagnosed at the Institute for Pathology and Molecular Pathology, University Hospital Zurich (Zurich, Switzerland) as described (10). Immunohistochemistry using rabbit anti-EVI1 antibodies (Cell Signaling, Danvers, MA) and digital expression analysis were performed as published (11). Briefly, a semi-quantitative image analysis software (Tissue Studio v.2, Definiens AG, Munich, Germany) was applied to digitalized TMA slides, obtaining a continuous spectrum of average brown staining intensity of tumor cell nuclei in arbitrary units. Subsequently, EVI1 expression was categorized in low, medium or high according to the 25th and 75th percentile of all measured expression values. Fluorescence *in-situ* hybridization (FISH) was used to detect *EVI1* copy number gains and rearrangements using the *EVI1*-flanking BAC clones CTD-2079P9 and RP11-264O10 for probe labeling (12).

Cell lines and culture

BC cell lines (DSMZ, Braunschweig, Germany) were bought in 2012 and re-authenticated by DSMZ in September 2014 and August 2015, respectively, using a nanoplex PCR for specific DNA profiles in 8 different highly polymorphic short tandem repeat (STR) loci. Additionally, samples were tested for the presence of

rodent mitochondrial DNA from mouse, rat, chinese and syrian hamster. Cells were cultivated according to data sheet. BC primary tissue samples were dissociated to single cells and cultured as described (4). Estradiol (Sigma-Aldrich, St Louis, MO), Kisspeptin-10 (Kp-10; Santa Cruz Biotechnology, Dallas, TX) and RKI-1447 (Selleckchem, Houston, TX) were used as indicated.

Lentiviral production and transduction

EVI1-specific or control shRNAs were designed using the MISSION TRC shRNA software tool and integrated into the pLKO.1-Puro vector system (Sigma) for lentiviral production. *EVI1* overexpression and control vectors (13) were kindly provided by Olga Kustikova and Christopher Baum (Hannover Medical School, Hannover, Germany). For inducible overexpression, *EVI1* or *KISS1* cDNAs (the latter cloned from MDA-MB-231 cells using primers as indicated in Supplementary Table S2) were integrated into a pLVX vector system to drive expression by doxycycline (Sigma) from a Tet_{on} lentiviral system (Clontech, Mountain View, CA). Lentiviral particles were produced and cells transduced as described (14).

siRNA treatment

Primary BC cells were cultured for 24 hours with a mixture of 3 independent siRNAs against EVI1 and respectively 2 control siRNAs (Life Technologies) together with lipofectamine (Invitrogen) in penicillin/streptomycin-free culture medium as described (14,15). Cells were then cultured under standard conditions for another 24 hours and then harvested for mRNA and functional assays.

RNA isolation, cDNA synthesis and real-time PCR

RNA was extracted with an RNeasy kit (Qiagen) and cDNA synthesized using a Thermo Script RT-PCR System (Invitrogen). Reverse transcripts were amplified by qRT-PCR and quantified upon incorporation of SYBR Green on an ABI 7500 workstation. Relative expression levels were calculated after normalization to the reference gene *GAPDH* using the $\Delta\Delta C_T$ method. *CDKN1A*, *CDKN1B*, *BIK* and *BBC3* primers were purchased from Qiagen (SYBR® Green QuantiTect Primer Assays). Other primer sequences are given in Supplementary Table S2.

Cell growth, cell cycle, proliferation and apoptosis assays

To assess cell growth, 50.000 cells were plated and quantified after trypsinization on days 3, 6 and 9 post-seeding. Cell proliferation was investigated by incorporation of BrdU or EdU as detailed in the manufacturers' protocols (BrdU: BD Biosciences, Heidelberg, Germany; Click-iT®, EdU kit: baseclick, Neuried, Germany). Cell cycle and apoptosis assays were performed as described (15). Cells were analyzed by flow cytometry for their DNA content on a FACS Fortessa machine using FlowJo software (FlowJo enterprise, Ashland, OR). For apoptosis assays, $5x10^4$ cells/ml were incubated overnight and then treated either for 16 h with staurosporine (2.5 µM; Sigma-Aldrich) or for 24 h with SuperKiller TRAIL™ (50 ng/ml; Enzo Life Sciences, Farmingdale, NY).

Immunoblotting

Immunoblotting was performed as described (4) using the following primary anti-

bodies (Cell Signaling): anti-pan AKT (#4691S), anti-pAKT (pSer473, #4060S), anti-ERK1/2 (#4695), anti-pERK1/2 (Thr202/Tyr204, #4377), anti-GAPDH (#5174P), anti-EVI1 (#2593), anti-p21 (#2947), anti-p27 (#3688), anti-CDK2 (#2546), and anti-β-actin (#3700S). Fluorescently labeled or HRP-conjugated secondary antibodies were used as described (14,15).

Microarray gene expression analysis

Microarrays analyses were performed in triplicates from control and *EVI1* knockdown MDA-MB-231 cells (obtained with either one of two independent *EVI1*-specific shRNAs). RNA was extracted with an RNeasy Mini kit (Qiagen). Concentration and purity of RNA samples were determined with a NanoDrop photometer (peqlab), and integrity confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNA samples with RIN values ≥ 7.5 were considered. Per condition, 100 ng of RNA were used to prepare cyanine-3-labeled cRNA for hybridizations, which were performed according to standard protocols using Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarrays. After extensive washing, fluorescence intensities were detected with the Scan Control A.8.4.1 software (Agilent) on an Agilent DNA Microarray Scanner and extracted from images using Feature Extraction 10.7.31 software (Agilent). Quantile normalization was applied to the data set and correlation analysis was performed. Fold change calculations identified differentially expressed genes, and Panther analysis most prominently affected pathways in *EVI1* knockdown vs. control cells. Array data will be deposited in the GEO database.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed as described (16). Briefly, $1x10^7$ control or *EVI1*-overexpressing Hs 578T cells were fixed in 1% formaldehyde, sonicated, pre-cleared and incubated with 10 µg anti-EVI1 or isotype control antibodies overnight at 4°C. Complexes were washed, DNA-extracted, precipitated and amplified by RT-PCR using primers sets homologous to regions of the human *KISS1* promoter. Non-immunoprecipitated chromatin was used as input control. Primers flanking the EVI1-binding site in the *BCL2L1* promoter and at a previously described non-binding site served as positive and negative control, respectively (17).

Migration assay

The established “wound healing assay” was performed to assess cell migration (18). Briefly, cells were grown to confluence in 24-well plates and incubated with 5 µg/ml aphidicolin (Sigma-Aldrich, A4487) and reduced FCS concentrations (2%) to stall proliferation. Subsequently, the monolayer was injured with a pipette tip and detached cells removed by iterative washing, leaving an approximately 200-µm wide unsettled zone free for lateral repopulation. Migration into these “wound areas” was followed on an Axio Vert.A1 microscope (Zeiss) and quantified by Fiji Imaging software at 0 h, 12 h and 24 h of incubation with or without addition of doxycycline, Kp-10 or RKI-1447 as indicated.

Zebrafish xenograft experiments

Animal experiments and zebrafish husbandry were approved by the “Kantonales Veterinaeramt Basel-Stadt”. Xenotransplantation and assessment of tumor cell engraftment were performed as described (4,19). In brief, 75 to 100 BC cells labeled with the fluorescent CellTracker™ (Life Technologies) were micro-injected at 48 h post fertilization into the vessel-free area of the yolk sac of transgenic Tg(flk1:eGFP) zebrafish embryos anesthetized in 0.4% tricaine (20). For rescue experiments, the fish water was supplemented with 100 nM estradiol (Sigma) or carrier (DMSO) at day 0 and 2.5 post transplantation. Tumor development was assessed microscopically at day 5 post-injection (19,21). For pERK inhibition, the fish water was supplemented with 200 nM of CI-1040 at days 1 and 2 post-transplantation.

Mouse xenograft experiments

NOD.Cg-Prkdc^{scid} IL2rg^{tmWjl}/Sz (NSG) mice purchased from Jackson Laboratory (Bar Harbor, ME) were maintained under pathogen-free conditions according to federal and state regulations. Control and EVI1 knockdown MDA-MB-231 cells ($1x10^6$) mixed with Matrigel (1:1, BD Biosciences) were co-laterally implanted by sub-cutaneous injection into the flanks of individual mice and occurrence of tumors monitored by palpation as reported (15). Tumor area was assessed *in situ* using the Fiji software and tumor weight was measured after excision.

Statistical analyses

Unless otherwise indicated, data from ≥3 independent biological experiments performed in technical triplicates were analyzed. Results are shown as mean ± SD. P-values were calculated by two-tailed, unpaired student's T-tests or as specified and p-values indicated with * for <0.05, ** for <0.01 and *** for <0.001. Retrospective survival analyses of BC patients were performed by the Kaplan-Meier method using log-rank (Mantel–Cox), Breslow and Tarone-Ware tests.

Results

EVI1 gene and protein expression in human breast carcinoma

First, we assessed *EVI1* gene and protein expression in 12 primary human BC samples (Fig. 1A) and 8 BC cell lines (Fig. 1B and C; ER-: MDA-MB-231, BT-549, Hs 578T, MDA-MB-468, SK-BR-3, and ER+: BT-474, T-47D, MCF7). EVI1 expression was detected in several samples irrespective of the ER status. To cover a comprehensive range of endogenous EVI1 expression for subsequent functional investigations, two ER- (MDA-MB-231 and Hs 578T) and two ER+ BC cell lines (T-47D and MCF7) were chosen and investigated alongside with four primary patient-derived cell samples of different ER status (P1-P4).

Furthermore, we employed immunohistochemistry to investigate EVI1 protein expression on a tissue microarray (TMA) of 608 BC samples (10). Reliable and biologically interpretable results were obtained from 527 samples, in 512 of which information on ER status was available. Consistent with our previous data, EVI1 protein was detected at variable degrees (Fig. 1 D) in both ER- (n=91) as well as ER+ (n=421) tumors (Fig. 1D and Supplementary Table S1). However, a significant correlation between EVI1 expression levels and survival was only observed in the ER- subgroup (n=91 patients; 5-year survival: p=0.011, overall survival: p=0.026) but not in the ER+ subgroup (n=421 patients) or the whole patient cohort analyzed together (Supplementary Fig. S1A). Interestingly, the influence of EVI1 expression on overall survival was most pronounced in triple-negative BC (p=0.006), but lost when ER-/HER2+ subsets were separately analyzed (Sup-

plementary Fig. S1A). Together, these data suggest that EVI1 expression is of particular significance in BC that is not driven by active ER and HER2 signaling.

ER- and triple-negative BC subgroups, in which EVI1 showed prognostic relevance, were subjected to further analysis of clinico-pathological parameters. High EVI1 expression associated indeed with enhanced distant metastasis rate (p=0.046 and p=0.027, respectively; Supplementary Fig. S1A), indicating a putative functional contribution to tumor cell dissemination/migration. To investigate the mechanisms responsible for EVI1 overexpression, we performed FISH analyses. Unlike in leukemia (22), we could not detect *EVI1* gene rearrangements or copy number gains except in two out of 512 BC patients (Fig. 1E, Supplementary Fig. S1B).

Based on these data we conclude that EVI1 expression is frequently observed in human BC, where it is mostly driven by yet unknown regulatory events, and might be particularly relevant for estrogen-independent HER2-negative tumors.

EVI1 induces cell proliferation and apoptosis resistance

To examine the functional significance of *EVI1* in BC, we performed *EVI1* knockdown experiments in two ER- (MDA-MB-231 and Hs 578T) and one ER+ (T-47D) BC cell line and two patient-derived primary BC samples per condition (ER+: P1, P2; ER-: P3, P4). Cells were transduced with lentiviral particles carrying either non-coding or two alternate *EVI1* shRNAs. Transduction with either shRNA

down-regulated EVI1 protein and mRNA expression when compared to controls (Fig. 2A, Supplementary Fig. S2A-B and S3A). Throughout all analyzed samples, *EVI1* knockdown cells showed a clear growth defect when compared to corresponding control cells (Fig. 2B, Supplementary Fig. S2C and S3B).

The lower growth rates observed in *EVI1* knockdown cells could be caused by decreased proliferation or enhanced apoptosis rates, both of which are modulated by EVI1 in other cell types (22). Indeed, knockdown of *EVI1* enhanced basal BC cell apoptosis (Fig. 3A, Supplementary Fig. S2D and Fig. S3C) as well as apoptosis sensitivity in response to staurosporine or the death ligand TRAIL (Fig. 3B, Supplementary Fig. S3D). In addition, cell cycle analyses revealed a G1 to S phase transition defect upon *EVI1* knockdown (Fig. 3C, Supplementary Fig. S3E) indicating a proliferation defect. Supporting this notion, BrdU incorporation was also diminished (Fig. 3D, Supplementary Fig. S3F). In line, key checkpoint regulators that block G1 to S phase transition, such as the cyclin-dependent kinase inhibitors 1A and 1B ($p21^{Cip1}$ and $p27^{Kip1}$) were up-regulated in *EVI1* knockdown MDA-MB-231 cells, while their mutual downstream target CDK2 was decreased (Supplementary Fig. S4).

Stimulation of the ER pathway rescues pERK expression and growth in *EVI1* knockdown BC cells

Intriguingly, the profound growth-modulatory effects of EVI1 were observed independent of the ER status, which is in apparent contrast to the prognostic signifi-

cance of EVI1 expression especially in ER- BC patients. Indeed, the *in vitro* findings in ER+ BC cells could be biased by lack or reduced ER stimulation under standard cultivation conditions. Confirming this hypothesis, addition of estradiol greatly restored growth of *EVI1* knockdown ER+ T-47D but not ER- MDA-MB-231 BC cells (Fig. 4A). Consistently, estradiol fostered the incorporation of EdU in T-47D but not in MDA-MB-231 *EVI1* knockdown cells (Fig. 4B). We conclude that active estrogen signaling overrules EVI1-mediated growth effects and, therefore, EVI1-mediated growth induction may be more critical for patients with ER- tumors that do not equally respond to natural estrogen.

ERK and AKT kinases are key regulators of cell proliferation and survival downstream of estrogen signaling (23-25). We thus wondered whether EVI1 also acts via activation of these kinases in BC. No consistent pAKT suppression was observed in *EVI1* knockdown cells (Fig. 4C and 4F), although *EVI1* overexpression indeed induced pAKT levels (Fig. 4D). However, an overt decrease in phosphorylated (i.e. activated) ERK levels was reproducibly noted upon *EVI1* knockdown throughout the analyzed ER- and ER+ BC samples (Fig. 4C and Supplementary Fig. S5A), indicating the ERK pathway as a dominant growth axis regulated by EVI1. Indeed, treatment with MEK inhibitors (CI-1040, trametinib or AZD6244) that act upstream of ERK (26) similarly suppressed cell growth and cycle progression of MDA-MB-231 and T-47D cells (Supplementary Fig. S5B-F). Further supporting this notion, addition of estradiol enhanced ERK but not AKT phosphorylation in *EVI1* knockdown ER+ T-47D but not ER- MDA-MB-231 BC cells

(Fig. 4C). *EVI1* overexpression consistently up-regulated pERK in MCF7, T-47D and MDA-MB-468 cells, and displayed synergistic effects with estradiol in ER+ T-47D cells (Fig. 4D-E). Interestingly, the rescue of cell growth induced by β-estradiol in *EVI1* knockdown cells was abrogated by co-treatment with either the ER blocking reagent tamoxifen or the MEK inhibitor CI-1040 (Supplementary Fig. S5G).

As also HER2 mediates growth-stimulatory effects via the MAPK/ERK signaling axis in BC, we further examined the significance of *EVI1* knockdown on HER-dependent ERK phosphorylation and found that, while loss of EVI1 signaling effectively depleted pERK in HER2- BC cells, ERK phosphorylation remained essentially unaltered in the investigated HER2+ samples (Fig. 4F, left vs. right panels). Together, these data suggest that EVI1, ER, and HER2 signaling functionally impinge on phosphor-modulation of ERK as a common downstream pathway.

EVI1* knockdown suppresses tumor formation *in vivo

Next, we used xenotransplantation assays to examine the relevance of *EVI1* for *in vivo* tumorigenesis from human BC cells. Equal numbers of *EVI1* knockdown and control MDA-MB-231 cells (ER-HER2-) were injected subcutaneously into contralateral flanks of immuno-permissive NSG mice and tumor formation was assayed over time. At 12 days post-transplantation, smaller tumors were documented from *EVI1* knockdown cells versus control cells (Fig. 5A-C), indicating that *EVI1* influences *in vivo* tumorigenicity. Immunoblot analysis confirmed per-

sistent knockdown of *EVI1* and impaired phosphorylation of ERK in excised tumors (Supplementary Fig. S6A). These data where confirmed in a previously established zebrafish xenotransplant model (4,19). Consistent with the results obtained in mouse, both *EVI1* knockdown ER+ T-47D and ER- MDA-MB-231 cells induced fewer tumors than corresponding control cells, while estrogen supplementation rescued *in vivo* tumor formation selectively from ER+ cells (Fig. 5D-E, Supplementary Fig. S6B). Moreover, the MEK inhibitor CI-1040 was able to block the rescue effect of β-estradiol *in vivo* (Supplementary Fig. S6C). In line with their reduced *in vivo* tumorigenicity, *EVI1* knockdown cells also displayed impaired mammosphere formation *in vitro* (Supplementary Fig. S6D). These data indicate that, although *EVI1* may not be a specific CSC marker in BC, it co-regulates the stem cell compartment.

Identification of GPCR signaling-associated molecules as *EVI1* downstream targets

To further explore the molecular mechanisms underlying *EVI1*-driven effects in BC, we analyzed the transcriptome of control and *EVI1* knockdown MDA-MB-231 cells using gene expression microarrays. 816 differentially expressed genes were identified in *EVI1* knockdown versus control cells, of which 324 were up- and 492 down-regulated. Panther analysis identified cell(-cell) adhesion, cell communication, signal transduction, developmental and immune system process regulation as the predominantly influenced biological processes, and receptors, cell-adhesion and respectively extracellular matrix proteins as the most significantly

affected protein classes (Fig. 6A). Furthermore, GeneSpring analyses revealed “G protein–coupled receptor (GPCR) signaling” molecules such as *KISS1, EDN1*, *PTGFR* and *PIK3CG* (Fig. 6B-C) as the most influenced pathway, next to cell-cycle control and progression (with perturbed expression levels of several key regulators such as *CDKN1A*, *CDKN1C*, *CCNA1* and *CDK1*), apoptosis resistance (with up-regulation of pro-apoptotic genes such as *BIK*, *BMF*, and *BBC3*) and ERBB signaling-related molecules (e.g. *EREG*, *DUSP5*, and *NRG2*). Heat maps of these individual categories are depicted in Fig. 6C and Supplementary Fig. S7A-B with a cut-off of 2-fold and 1.5-fold expression changes, respectively. *EVI1*-dependent expression changes of 15 exemplary candidate genes were further validated by qRT-PCR (Supplementary Fig. S7C).

To identify potential direct target genes of *EVI1* in BC, we next investigated the expression of candidate genes in response to *EVI1* overexpression (Supplementary Fig. S7D). Among these, the GPR54-ligand *KISS1* stood out as one of the most strongly influenced genes. Additionally, the induction of *KISS1* mRNA displayed a clear dose-dependency on *EVI1* transcript levels (Fig. 7A). Furthermore, co-depletion of *EVI1* and *KISS1* mRNA was observed in primary BC cells treated with siRNAs against *EVI1* versus corresponding control siRNA-treated cells (Supplementary Fig. S7E). Moreover, promoter analysis of *KISS1* revealed several potential EVI1-binding sites within *KISS1* regulatory elements (Supplementary Fig. S8A), reinforcing *KISS1* as a putative direct transcriptional target of EVI1. Based on this analysis, four promoter regions of *KISS1* were selected

(Supplementary Fig. S8A) and assessed for EVI1 binding in chromatin immunoprecipitation (ChIP) assays. Higher enrichment rates were indeed observed in EVI1-overexpressing vs. control cells especially at the most distal promoter site (-4880 to -4761 bp, Fig. 7B). Thus, these data identify the *KISS1* promoter as a yet unrecognized target region for EVI1 in BC (see also Supplementary Fig. S8 for control and schematic illustration of binding sites). We therefore conclude that, next to modulating expression of cell cycle- and apoptosis-relevant genes (Fig. 6B-C and Supplementary Fig. S7B-C), EVI1 directly influences GPCR signaling via transcriptional modulation of the GPR54 ligand KISS1.

Differential role of KISS1 in EVI1-mediated cell migration, cell growth and ERK activation

KISS1 was originally identified as a metastasis suppressor (27,28), and more recently described to enhance motility and invasiveness of ER- BC cells (29,30). We thus hypothesized that EVI1 contributes to these processes at least in part via transcriptional regulation of KISS1. Indeed, migration assays revealed that knockdown of EVI1 strongly impaired the motility of ER- MDA-MB-231 (Fig. 7C-F) and Hs 578T cells (Supplementary Fig. S9A-B), whereas overexpression of EVI1 overtly increased cell mobility (Fig. 7G-H, Supplementary Fig. S9C-D). Supporting the role of KISS1 as a downstream target in EVI1-dependent migration, exposure of cells to the GPR54-ligand Kisspeptin-10 (Kp-10), a gene product of KISS1 shown to enhance ER- BC cell motility (Supplementary Fig. S10A), indeed rescued the migration defects observed in *EVI1* knockdown MDA-MB-231

cells (Fig. 7C-D). Supporting these data, overexpression of KISS1 itself also rescued migration in EVI1 knockdown MDA-MB-231 (Supplemental Fig. S10A-B and Fig. 7E-F) and Hs 578T cells (Supplemental Fig. S9A-B).

Noteworthy, several further modulators of cell motility and adhesion were found to be regulated by EVI1 in our microarray analysis, including e.g. RhoJ and TIE1, two molecules that had not been linked to EVI1 or BC cell migration before. Exemplifying the functional relevance of also these findings, inhibition of RHO/ROCK signaling with RKI-1447 impaired EVI1-induced BC cell mobility in migration assays (Fig. 7G-H and Supplementary Fig. S9C-D).

Interestingly, supplementation with Kisspeptin (Kp-10), which effectively rescued migration (Figure 7C-D), could not restore cell growth in *EVI1* knockdown cells (Supplementary Fig. S10C). Consistently, neither treatment with Kisspeptin (Kp-10) nor KISS1 overexpression influenced pERK activity (Supplementary Fig. S10D). Vice versa, treatment of BC cells with the MAPK inhibitor CI-1040 effectively suppressed pERK and cell growth (Supplementary Fig. S5F and Supplementary Fig. S5B-C) but did not influence BC cell migration (Supplementary Fig. S10F-G). In particular, treatment with CI-1040 did not abrogate EVI1-induced KISS1 overexpression (Supplementary Fig. S10E) and related BC cell migration, reinforcing the idea that these effects are independent of the MAPK pathway (Fig. 7I).

Taken together, we demonstrate a functional relevance of *EVI1* gene expression for BC cell growth that involves modulation of pERK signaling (see Fig. 7I for schematic illustration). In part complementary to ER signaling and eventually overruled by constitutive ERK activity in HER2+ BC, EVI1-mediated effects achieve pivotal significance in ER- HER2- BC, where EVI1 expression is of prognostic significance. Moreover, we present evidence for a hitherto unrecognized EVI1-KISS1-GPR54 axis that – independently of ERK signaling – modulates BC cell motility, suggesting that also the capacity to induce metastasis may be intimately linked to EVI1. Thus, our work identifies EVI1 as a novel critical determinant of BC cell biology that is of particular importance for estrogen-independent HER2- BC.

Discussion

Initially identified as a retroviral insertion region in hematopoietic cells (31), EVI1 has been intensely studied in HSCs and AML where it represents a marker of adverse prognosis. EVI1 is also expressed in other tissues such as brain, lung, and kidney (32-34). Pointing towards its significance in early organogenesis, *Evi1* knockout mice are embryonically lethal and show broad hypocellularity and patterning defects (35,36). The molecular regulation and functional relevance of EVI1 expression in BC however are largely unexplored.

Analysis of a large cohort of primary samples did not detect significant gene rearrangements or copy number gains, indicating that activation of the *EVI1* locus in BC follows different principles than in myeloid leukemia, such as regulation via miRNAs (8). Consistently, a common *EVI1* polymorphism (rs6774494 A>G) targeted by miR-206/133b was suggested to predict adverse outcome in postmenopausal BC patients (37). Interestingly, immunohistochemical analyses of our patient cohort identified EVI1 protein as a prognostic marker in ER- but not ER+ BC, supporting previous mRNA-based studies (8,38). Importantly, when the ER- cohort was further subdivided in ER-HER2+ and triple-negative BC, EVI1 expression influenced survival specifically in the latter. In this subgroup, high EVI1 expression further associated with enhanced distant metastases.

Functional studies documented a profound growth defect in *EVI1* knockdown versus control cells, resulting from impaired proliferation, cell cycle progression

and apoptosis resistance. Interestingly, addition of estrogen to ER+ but not ER- cells restored the impaired ERK activation and proliferation. Furthermore, both effects of β-estradiol were abrogated by co-treatment with either the ER blocking reagent tamoxifen or the MAPK inhibitor CI-1040, which highlights the specificity of the observed effects and indicates that EVI1 and β-estradiol merge in pERK activation to regulate BC cell growth. However, an inverse correlation was documented between EVI1 expression and tumor size in triple-negative BC and by trend also in the ER- subgroup. We hypothesize that the subgroup of BC presenting with large primary tumor size and negative to low EVI1 expression is driven by aggressive, yet EVI1-unrelated molecular mechanisms.

Our analyses reliably identified the significance of EVI1 expression in the absence of endogenous estrogen signaling. In contrast, in the ER+ BC cohort such analyses might be complicated by the fact that these patients receive anti-estrogen treatments. The importance of EVI1 expression might differ depending on the patient response and efficacy of such treatments. Our functional data show that EVI1 also severely regulates the growth of ER+ BC cells, if estrogen is not provided. Unfortunately, we have no detailed and complete information on the mode and efficacy of anti-estrogen treatments of the ER+ BC patients. Thus, while our analyses support the notion that EVI1 could impact BC independently of ER signaling, assessment of the relevance of EVI1 in ER+ BC requires further patient stratification according to the response to anti-estrogen treatment.

Suppression of EVI1 expression consistently inhibited MAPK activation in HER2- but not HER2+ BC. Thus, potential inhibitory effects of *EVI1* knockdown on MAPK signaling might be overruled by constitutive HER2 activity. This assumption is consistent with the results in patients where EVI1 expression levels were prognostically relevant in triple-negative BC, but not in ER- HER2+ subsets. Thus, EVI1, estrogen and HER2 signaling might converge on MAPK signaling as a common downstream effector controlling BC cell growth. Growth-stimulatory properties of estrogen in BC also involve transcriptional induction of cyclin D1 (39) and suppression of CDK inhibitors, such as $p21^{Cip1}$ or $p27^{Kip1}$ (40). Indeed, our investigations uncovered that also these growth-regulatory events are influenced by EVI1 and, moreover, that EVI1 modulates the expression of several further key cell cycle regulators (e.g. CDKN1A, CDKN1B, CDKN1C, CCNA1 and CDK1).

In addition, our data suggest that EVI1 enhances apoptosis resistance in BC by inducing a concerted suppression of pro- and induction of anti-apoptotic genes. In line with previous data (14,17), we found a physical association of EVI1 with the *BCL-XL* promoter. Previous links between EVI1 and apoptosis include direct blocking interactions with JNK in hematopoietic cells, and the inhibition of apoptosis through a PI3K-dependent mechanism in colon cancer cells (41,42). EVI1 is further discussed as a stem cell factor in hematopoiesis and leukemia (22), but in BC it rather homogenously marked tumor cells, at least in cases of high EVI1 expression. Nevertheless, *EVI1* knockdown impacted the frequency of *in vivo*

tumor- as well as *in vitro* mammosphere-initiating BC cells. These data suggest that, even though not confined to breast CSCs, EVI1 expression might also regulate this compartment.

Intriguingly, we identified migration as a novel cellular function promoted by EVI1 in BC. In particular, *EVI1* knockdown impaired the BC cell mobility, whereas its overexpression enhanced migration. Gene microarray and qRT-PCR experiments surprisingly uncovered, next to regulators of cell cycle and apoptosis, several factors implicated in cell communication and GPCR signaling as downstream effectors of EVI1. Of these, we analyzed in more detailed the GPR54-ligand KISS1, which ChIP assays identified as a novel transcriptional target of EVI1. The EVI1-KISS1 ligand axis promoted motility of ER- BC cells, in line with previously reported roles of KISS1 on mobility and adhesion in this disease entity (29,30). Interestingly, although KISS1 has been reported as an upstream regulator of ERK (43), stimulation with the GPR54-ligand Kp-10 was not able to restore pERK and proliferation of EVI1 knockdown BC cells, although it did influence BC cell migration. Vice versa, MAPK inhibition effectively suppressed cell growth but did not alter migration, again reinforcing the idea that the EVI1-KISS1 migratory axis acts independently of pERK. Besides KISS1, we identified additional established (e.g. CXCR4, CCR1, AKAP12) (44-46) as well as novel factors in BC cell migration as targets of EVI1. For instance, TIE1, a modulator of angiogenesis and cell adhesion (47), and RhoJ, a modulator of the RHO/ROCK-dependent cell motility (48), were found to be modulated by EVI1, suggesting that this transcrip-

tion factor serves as a master regulator of BC cell motility.

Taken together, our data identify EVI1 as a potent oncoprotein regulating BC cell proliferation, apoptosis resistance and migration. Interestingly, estrogen and HER2 signaling as well as EVI1-mediated transcriptional modulation seemingly merge to stimulate MAPK signaling. This functional convergence identifies EVI1 as a major driver of cell growth acting independently of estrogen signaling. EVI1 and downstream MAPK activation might represent therapeutic targets in patients suffering from HER2- ER- or ER+ BC resistant to anti-estrogen therapies. Finally, targeting the newly identified EVI1-GPR54-KISS1 axis, for example by GPR54 inhibitors, may be considered for the treatment of metastasizing ER- BC. Effective targeting of EVI1-induced BC cell migration might however require either inhibition of EVI1 itself or joint suppression of additional migratory pathways (e.g. RHO/ROCK signaling).

Acknowledgments

We thank Olga Kustikova and Christopher Baum for provision of *EVI1* plasmids, and Joelle Müller for technical support in zebrafish experiments.

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Figure legends

Figure 1: Differential expression of *EVI1* in human BC cells. **A,** qRT-PCR analysis of *EVI1* expression in 12 primary BC samples and **B,** 8 BC cell lines. Indicated are *EVI1* expression levels relative to MDA-MB-231 cells (dotted line); midline illustrates average expression (A). **C,** Immunoblots documenting variable degrees of EVI1 protein expression in ER+ and ER- BC cell lines. Predominant isoforms (MDS/EVI1, EVI1, and EVI1Δ) are indicated. Beta-actin is shown for loading control. **D,** Immunohistological image sections illustrating different degrees (weak, medium, strong) of EVI1 expression in BC TMA samples. Overview pictures (bottom, scale bars = 100 µM), inlays at higher magnification (top). **E,** Representative FISH analysis showing normal distribution of EVI1 copy numbers in BC. Red and green FISH probes, respectively, flank the *EVI1* gene locus. Nucleus (DAPI, blue).

Figure 2: *EVI1* promotes BC cell growth *in vitro*. **A,** Immunoblot and qRT-PCR analyses documenting efficient depletion of EVI1 expression in *EVI1* knockdown versus control BC cell lines and primary patient-derived cell samples. ER- (MDA-MB-231, P3 and P4, left), ER+ (T-47D, P1 and P2, right). Due to low protein levels in patient sample P4, depletion of EVI1 expression was confirmed by RT-PCR. **B,** Growth curves illustrating a proliferation defect in *EVI1* knockdown vs. control cells. ER- cells (left), ER+ cells (right). Plotted are mean values ± SD.

Figure 3: EVI1 affects apoptosis regulation and cell cycle progression in BC. *EVI1* knockdown MDA-MB-231 (left panels) and T-47D cells (right panels) reveal **A,** elevated basal apoptosis; **B,** increased apoptosis sensitivity in response to TRAIL and staurosporine; **C,** increased cell cycle arrest and **D,** reduced BrdU incorporation compared to control shRNA-treated cells. Shown are mean values ± SD.

Figure 4: EVI1 synergizes with estrogen and HER2 signaling in the activation of MAPK/ERK. **A,** Growth curves, **B,** EdU incorporation and **C,** immunoblots of pERK and pAKT performed on EVI1 knockdown versus control MDA-MB-231 and T-47D cell lines propagated in the absence or presence of estradiol (100 nM). **D,** Immunoblot analyses showing increased pERK and pAKT levels in *EVI1* overexpressing versus control MCF7, T-47D and MDA-MB-468 BC cells grown in the absence or presence of estradiol. **E,** EVI1 and estradiol synergize in the induction of pERK in T-47D cells. **F,** Knockdown of EVI1 expression depletes pERK from HER2- BC cells (MDA-MB-231, patient samples P3 and P4, left), but not from HER2+ cells (SKBR3, patient samples P1 and P5, right).

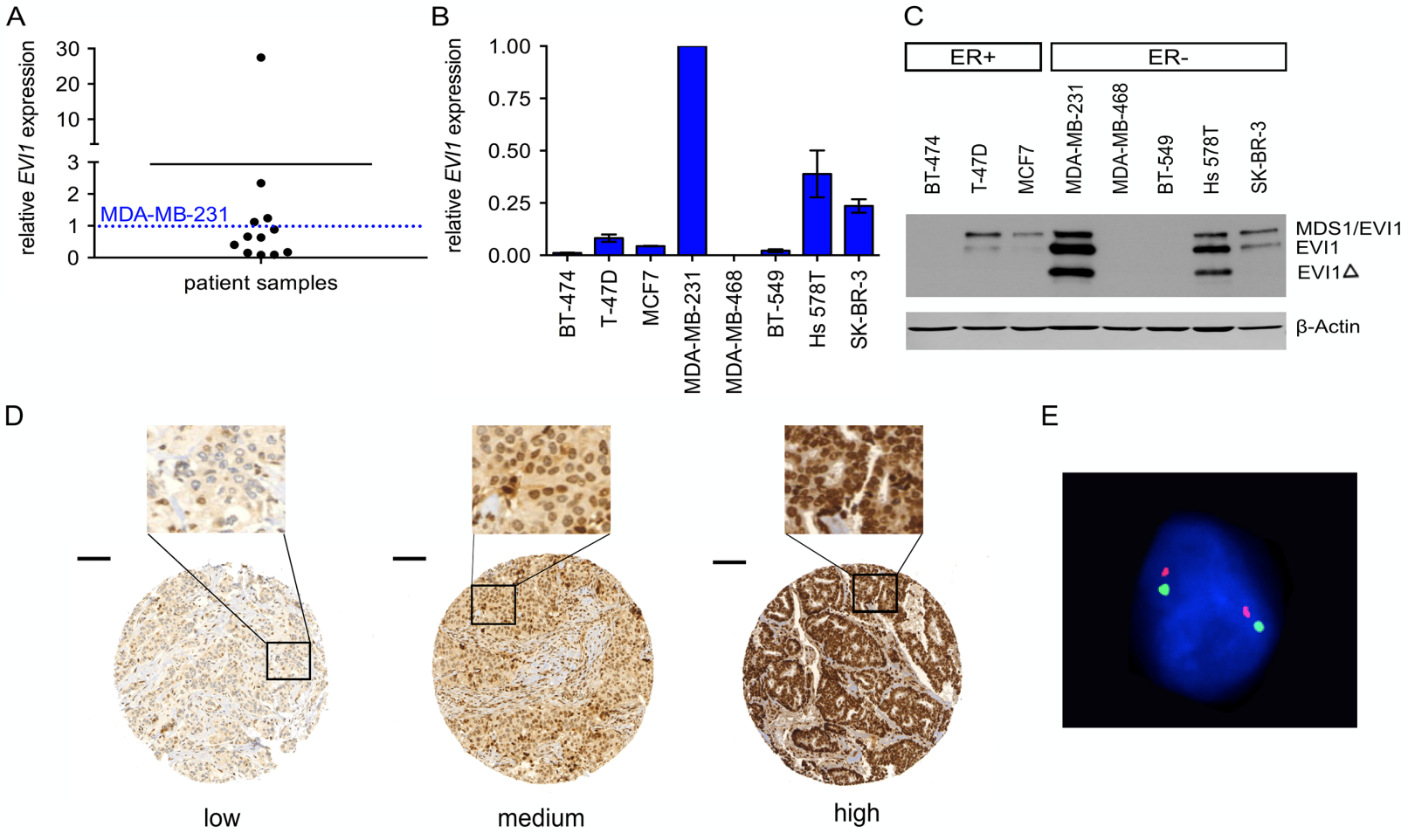
Figure 5: *EVI1* knockdown impairs tumor growth *in vivo*. **A,** ER- MDA-MB-231 control (right) and *EVI1* knockdown cells (left) were contra-laterally injected subcutaneously into NSG mice. Illustrated is a representative example of tumor formation after a follow-up of 12 days. Note that *EVI1* knockdown cells generate smaller tumors (left). **B/C,** Corresponding quantitative analysis of tumor area and

tumor masses (n=5). P-values were calculated by a Mann-Whitney test. **D,** 75-100 CM-DiI-labeled control or EVI1 knockdown BC cells were transplanted into the yolk sac of *Tg(kdrl:eGFP)* fish embryos and analyzed at day 5 post-transplantation by confocal microscopy for tumor formation (red). **E,** estradiol treatment (100 nM estradiol, 3 days pre-treatment of cells *in vitro* and afterwards added to the fish water) restores reduction of tumor formation upon *EVI1* knock-down in ER+ T-47D cells.

Figure 6: Gene expression patterns associated with *EVI1* knockdown. **A,** Panther classification linking gene signatures from *EVI1* knockdown transcriptome analysis to Biological Process and Protein Classes. Significantly modulated gene/protein classes are indicated. **B, I**ndex list of pathways significantly modulated by *EVI1* knockdown (i.e. $p<0.05$). **C**, Heat-maps depicting 24 individual gene entries whose expression significantly differs in a microarray analysis of control vs. *EVI1* knockdown MDA-MB-231 cells. Genes most strongly affected by *EVI1* knockdown functionally cluster in the categories cell cycle regulation, apoptosis, GPCR and ERBB signaling.

Figure 7: EVI1 regulates BC cell migration via modulation of GPR54/KISS1 and RHO/ROCK signaling. **A,** qRT-PCR analyses documenting dose-dependent co-induction of *EVI1* and *KISS1* in inducible *EVI1*-overexpressing T-47D cells. **B,** ChIP analysis illustrating direct recruitment of EVI1 to regulatory *KISS1* promoter elements in Hs 578T control and more strongly in *EVI1*-overexpressing cells. **C-F,**

Supplementation with the soluble *KISS1* gene product Kp-10 (1 µM, C-D), or overexpression of KISS1 itself (E-F) ameliorates migration defects imposed by EVI1 knockdown in MDA-MB-231 BC cells. Overview images (left), corresponding assay quantifications (right). **G-H,** Migration assays documenting increased mobility of MDA-MB-231 cells in response to EVI1 overexpression, whereas treatment with the RHO/ROCK pathway inhibitor RKI-1447 impairs the mobility of EVI1-overexpressing MDA-MB-231 cells. Image sections (G, left), corresponding assay quantification (H, right). **I,** Integrated scheme of *EVI1*-dependent signaling pathways influencing BC cell migration and growth.


A
relative EVI1 expression
30
20
10
3
2
1
0
MDA-MB-231
patient samples
B
relative EVI1 expression
1.00
0.75
0.50
0.25
0.00
BT-474
T-47D
MCF7
MDA-MB-231
MDA-MB-468
BT-549
Hs 578T
SK-BR-3
C
ER+
ER-
BT-474
T-47D
MCF7
MDA-MB-231
MDA-MB-468
BT-549
Hs 578T
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MDS1/EVI1
EVI1
EVI1Δ
β-Actin
D
low
medium
high
E

Figure. 1

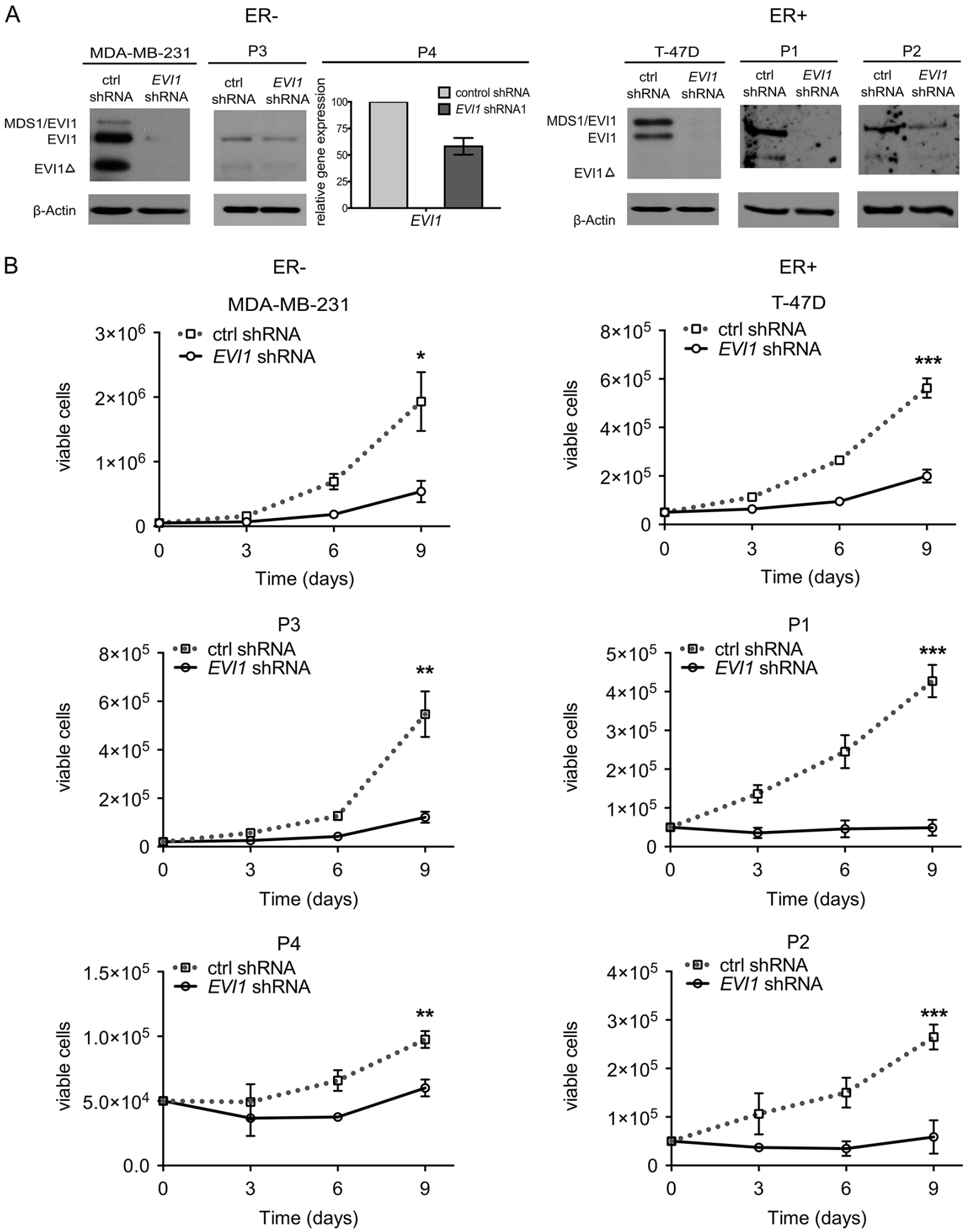

A
ER-
ER+
MDA-MB-231
P3
P4
T-47D
P1
P2
ctrl shRNA
EVI1 shRNA
MDS1/EVI1
EVI1
EVI1Δ
β-Actin
relative gene expression
control shRNA
EVI1 shRNA1
EVI1
B
ER-
ER+
MDA-MB-231
T-47D
P3
P1
P4
P2
ctrl shRNA
EVI1 shRNA
viable cells
Time (days)
*
**
***

Figure. 2

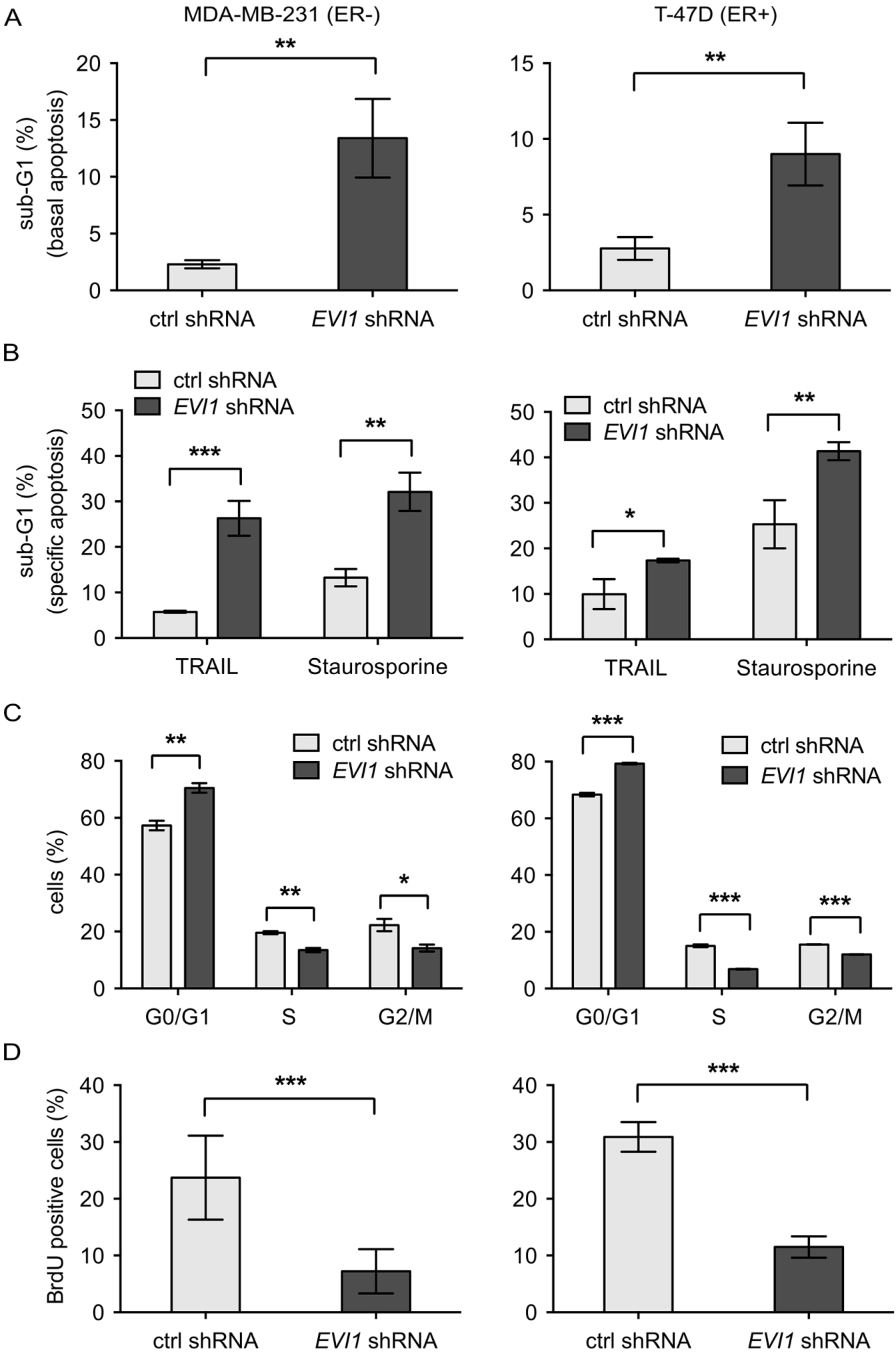

A
MDA-MB-231 (ER-)
T-47D (ER+)
sub-G1 (%)
(basal apoptosis)
**
**
ctrl shRNA
EVI1 shRNA
B
ctrl shRNA
EVI1 shRNA
sub-G1 (%)
(specific apoptosis)
***
**
*
**
TRAIL
Staurosporine
C
cells (%)
**
**
*
***
***
***
G0/G1
S
G2/M
D
BrdU positive cells (%)
***
***

Figure. 3

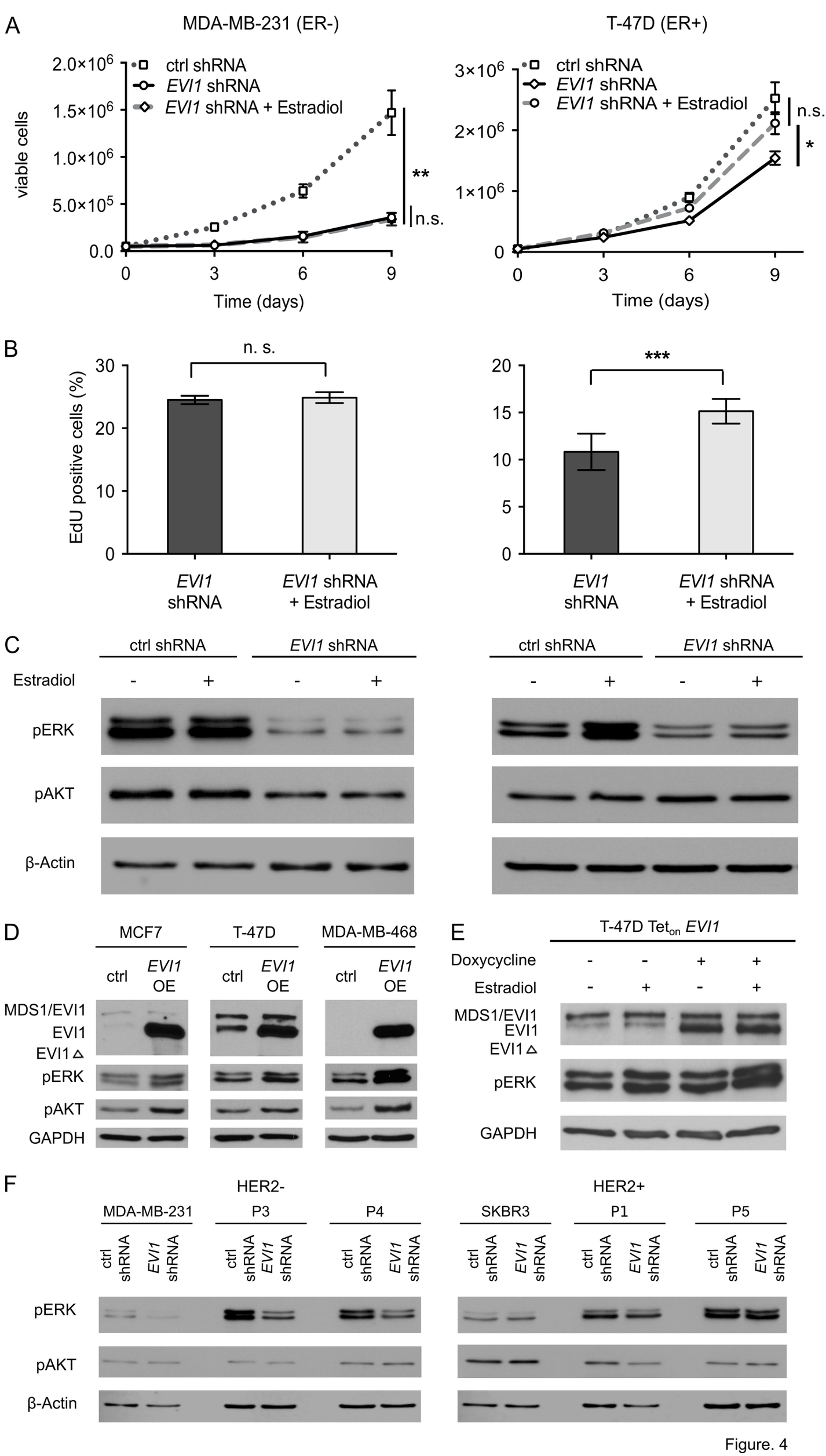

A
MDA-MB-231 (ER-)
T-47D (ER+)
ctrl shRNA
EVI1 shRNA
EVI1 shRNA + Estradiol
viable cells
Time (days)
**
n.s.
*
B
n. s.
***
EdU positive cells (%)
EVI1 shRNA
EVI1 shRNA + Estradiol
C
ctrl shRNA
EVI1 shRNA
Estradiol
pERK
pAKT
β-Actin
D
MCF7
T-47D
MDA-MB-468
ctrl
EVI1 OE
MDS1/EVI1
EVI1
EVI1△
pERK
pAKT
GAPDH
E
T-47D Tet$_{on}$ EVI1
Doxycycline
Estradiol
MDS1/EVI1
EVI1
EVI1△
pERK
GAPDH
F
HER2-
HER2+
MDA-MB-231
P3
P4
SKBR3
P1
P5
ctrl shRNA
EVI1 shRNA
pERK
pAKT
β-Actin

Figure. 4

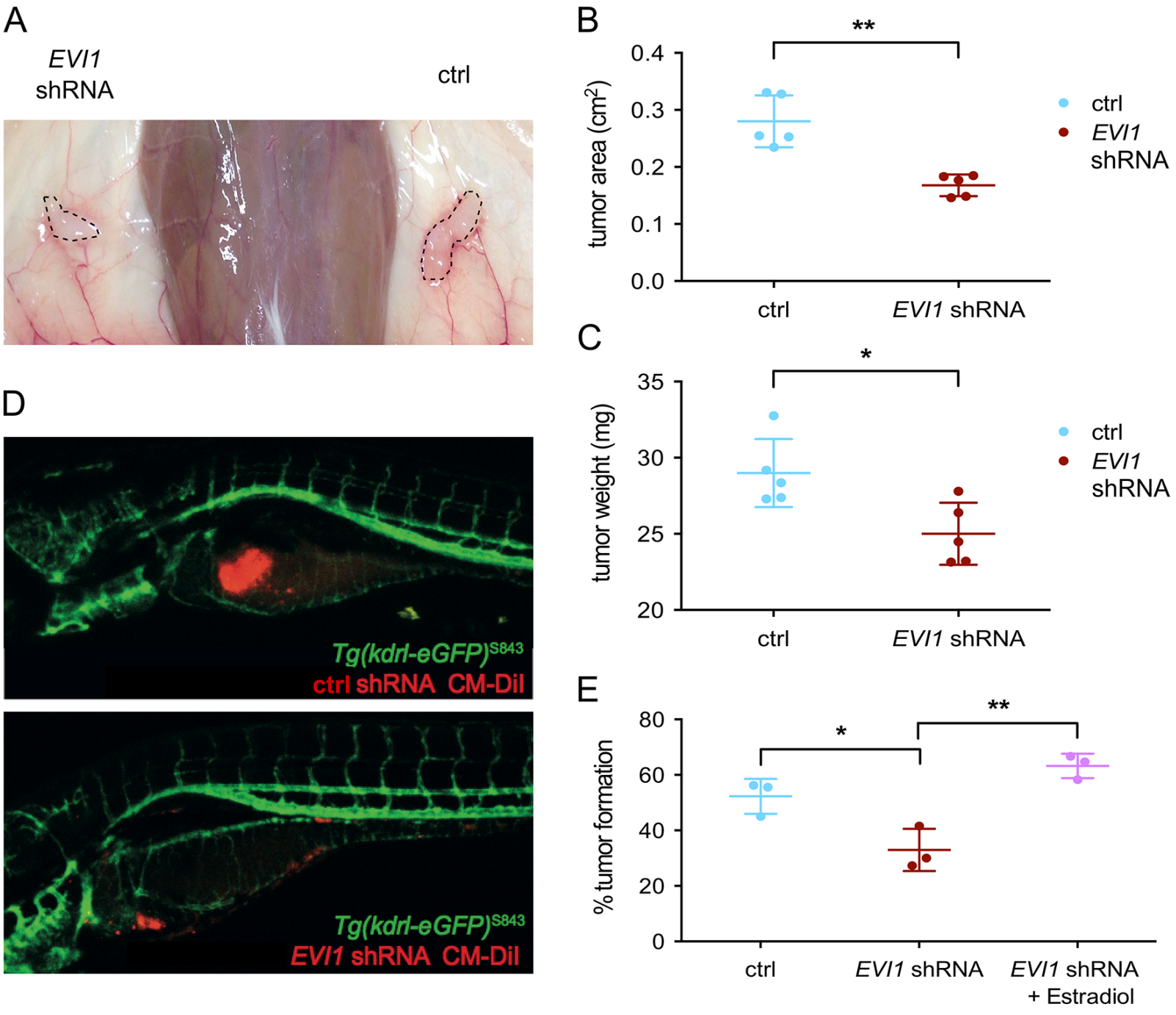
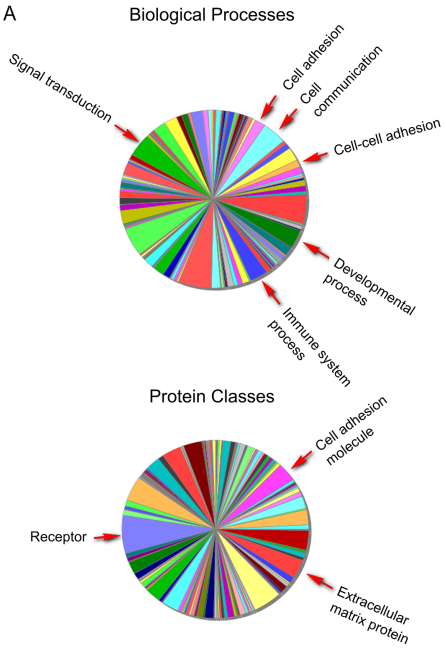

A
EVI1 shRNA
ctrl
B
**
tumor area (cm2)
0.4
0.3
0.2
0.1
0.0
ctrl
EVI1 shRNA
ctrl
EVI1 shRNA
C
*
tumor weight (mg)
35
30
25
20
ctrl
EVI1 shRNA
ctrl
EVI1 shRNA
D
Tg(kdrl-eGFP)S843
ctrl shRNA CM-DiI
Tg(kdrl-eGFP)S843
EVI1 shRNA CM-DiI
E
*
**
% tumor formation
80
60
40
20
0
ctrl
EVI1 shRNA
EVI1 shRNA
+ Estradiol

Figure. 5

A



B

	Pathway	p-value
Down		
	RB in Cancer	5.51E-06
	GPCR downstream signaling	2.34E-05
	Small Ligand GPCRs	3.07E-04
	GPCR ligand binding	4.38E-04
	Mitotic Prometaphase	1.34E-02
	Cell Cycle	2.08E-02
	DNA Damage Response	2.46E-02
	GPCRs, Class B Secretin-like	3.88E-02
	GPCRs, Class A Rhodopsin-like	4.12E-02
	Signal Transduction of S1P Receptor	4.33E-02
Up		
	Differentiation Pathway	1.64E-03
	ErbB Signaling Pathway	2.71E-03
	Apoptosis Modulation and Signaling	4.82E-03
	Signaling by ERBB2	5.65E-03
	Signaling by ERBB4	1.38E-02
	EGF-EGFR Signaling Pathway	2.54E-02
	GPCR ligand binding	2.75E-02
	Estrogen metabolism	4.35E-02

C

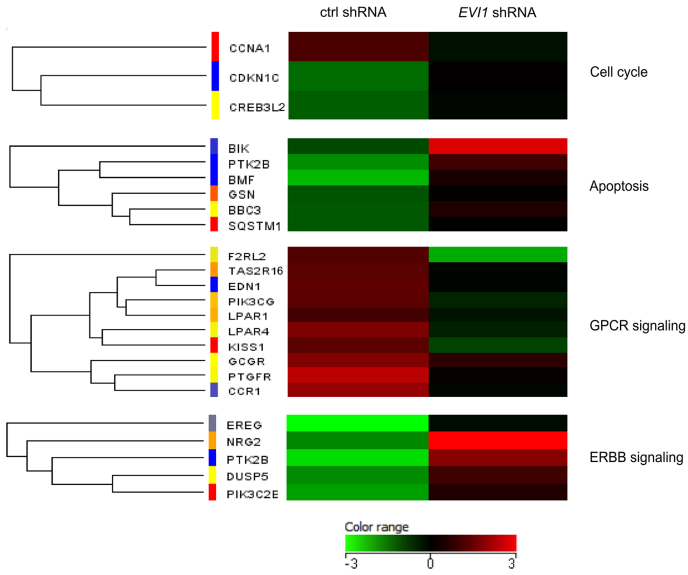


Figure. 6

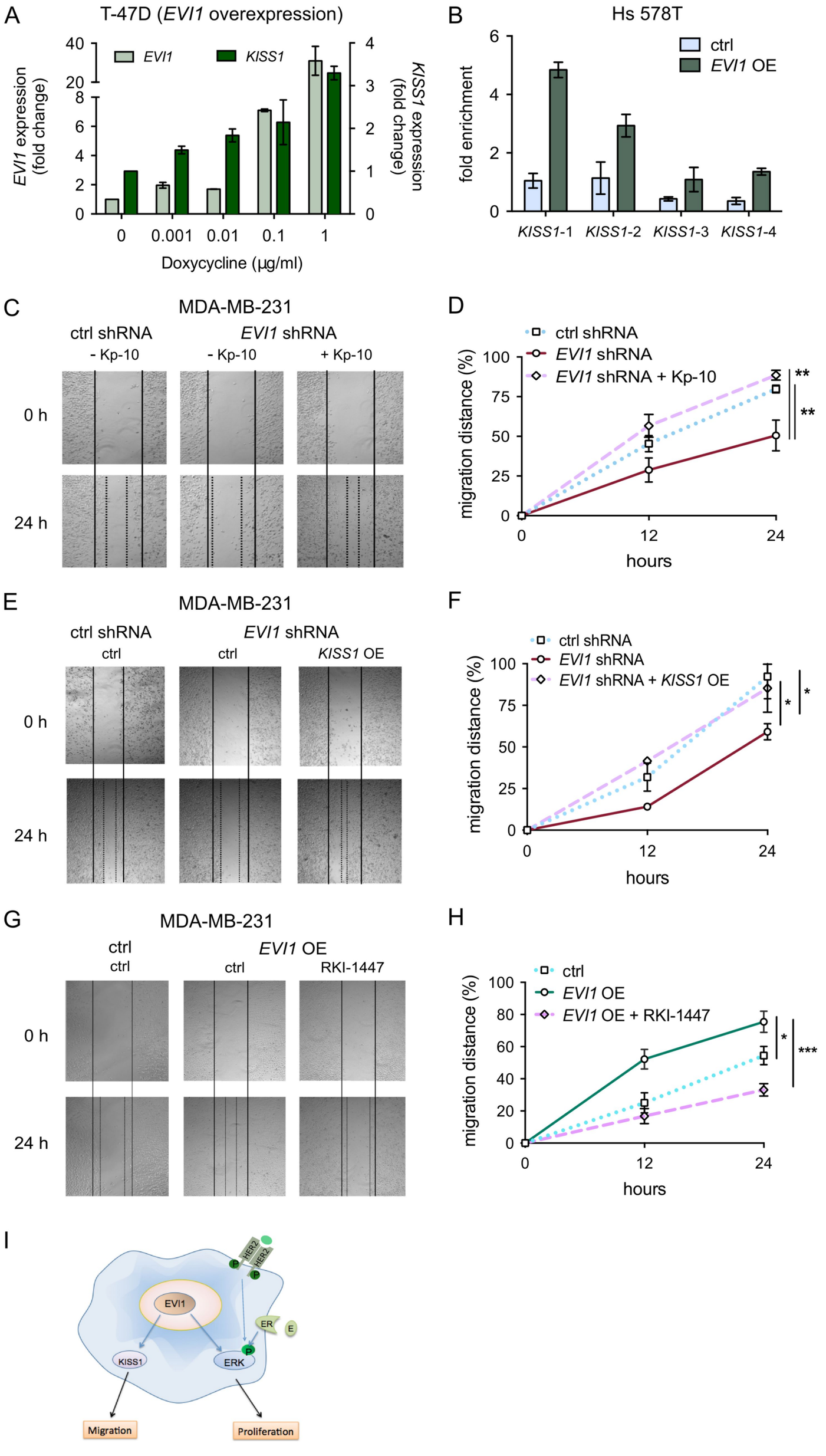

A
T-47D (EVI1 overexpression)
EVI1
KISS1
EVI1 expression (fold change)
KISS1 expression (fold change)
Doxycycline (µg/ml)
B
Hs 578T
ctrl
EVI1 OE
fold enrichment
KISS1-1
KISS1-2
KISS1-3
KISS1-4
C
MDA-MB-231
ctrl shRNA
EVI1 shRNA
- Kp-10
- Kp-10
+ Kp-10
0 h
24 h
D
ctrl shRNA
EVI1 shRNA
EVI1 shRNA + Kp-10
migration distance (%)
hours
E
MDA-MB-231
ctrl shRNA
EVI1 shRNA
ctrl
ctrl
KISS1 OE
0 h
24 h
F
ctrl shRNA
EVI1 shRNA
EVI1 shRNA + KISS1 OE
migration distance (%)
hours
G
MDA-MB-231
ctrl
EVI1 OE
ctrl
ctrl
RKI-1447
0 h
24 h
H
ctrl
EVI1 OE
EVI1 OE + RKI-1447
migration distance (%)
hours
I
HER2
HER2
P
P
EVI1
ER
E
P
KISS1
ERK
Migration
Proliferation

Figure. 7